PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 3852PTWO/er	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/EP2004/000183	International filing date *(day/month/year)* 14.01.2004	Priority date *(day/month/year)* 14.01.2003
International Patent Classification (IPC) or national classification and IPC C12N5/08		
Applicant SINTOFARM S.P.A. et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 7 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 3 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☒	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12.08.2004	04.04.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Sommer, B Telephone No. +49 89 2399-7099

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2004/000183

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-15 as originally filed

Claims, Numbers

1-28 received on 10.11.2004 with letter of 08.11.2004

Drawings, Sheets

1/5-5/5 as originally filed

☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2004/000183

Box No. II Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
   - ☐ copy of the earlier application whose priority has been claimed (Rule 66.7(a)).
   - ☐ translation of the earlier application whose priority has been claimed (Rule 66.7(b)).

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rule 64.1). Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

   see separate sheet

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application,

   ☒ claims Nos. 27, 28

   because:

   ☒ the said international application, or the said claims Nos. 27, 28 relate to the following subject matter which does not require an international preliminary examination (specify):

   see separate sheet

   ☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos.

   ☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form	☐	has not been furnished
	☐	does not comply with the standard
the computer readable form	☐	has not been furnished
	☐	does not comply with the standard

   ☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

   ☐ See separate sheet for further details

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-28
	No:	Claims	-
Inventive step (IS)	Yes:	Claims	1-28
	No:	Claims	-
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	-

2. Citations and explanations (Rule 70.7):

see separate sheet

Re Item II

The current assessment is based on the assumption that all claims enjoy the priority rights from the filing date of the priority document (14.01.2003)

Re Item III

Claims 27 and 28 relate to subject-matter considered by this Authority to be covered by the provisions of **Rule 67.1(iv) PCT**. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (**Article 34(4)(a)(I) PCT**). For the assessment of the present claims 27 and 28 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item V

1. Reference is made to the following documents (D):

D1: CORADINI D ET AL: 'Hyaluronic acid as drug delivery for sodium butyrate: improvement of the anti-proliferative activity on a breast-cancer cell line' INT. J. CANCER, vol. 81, no. 3, 5 May 1999, pages 411-416

D2: MCBURNEY ET AL: 'Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line' NATURE, vol. 299, 9 September 1982, pages 165-167

D3: WOBUS AM ET AL: 'In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid' ROUX'S ARCH. DEVELOP. BIOL., vol. 204, 1 October 1994, pages 36-45

D4: WOBUS AM ET AL: 'Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes' J. MOL. CELL. CARDIOL., vol. 29, 1997, pages 1525-1539

D5: XU C ET AL: 'Characterization and enrichement of cardiomyocytes derived from human embryonic stem cells' CIRC. RES., vol. 91, 2002, pages 501-508

2. The present application concerns the use of retinoic or retinoic/butyric esters of hyaluronic acid for inducing the differentiation of stem cells into cardiomyocytes. The preparation of cardiomyocytes, a process of screening for cardiogenic compounds, an *in vitro* model for cardiogenic differentiation and therapeutic methods are claimed.

3. The subject-matter of claims 1-28 appears novel in the sense of **Article 33(2) PCT.**

4. D3, which is considered as closest prior art, teaches that a treatment with 10^{-9} to 10^{-7}M retinoic acid between the 5th and 7th day of embryonic body formation induces cardiogenesis in pluripotent embryonic stem (ES) cells. When retinoic acid is given outside this time window or at different concentrations, the embryoid bodies develop to skeletal myocytes and/or neuronal cells while cardiogenesis is completely inhibited (D3, e.g. abstract, results, discussion).

The technical problem underlying the present application seems to be the provision of a further method for inducing cardiogenesis in an embryonic cell line. The application solves this technical problem by using hyaluronic acid esters of retinoic acid and optionally butyric acid to achieve the differentiation of stem cells into cardiomyocytes.

Both retinoic acid as well as butyrate are known to induce the differentiation of pluripotent embryonic cells into cardiomyocytes (e.g. D2, e.g. page 167, left-hand column, paragraph 3-4; table 1; D3, e.g. abstract, results, discussion; D4, e.g. abstract, discussion). However, the cardiogenesis-inducing effect of retinoic acid on murine embryonic cell lines is limited to a specific time of addition and to particular concentrations (e.g. D3, abstract; results; discussion). In human ES cells, treatment with retinoic acid was toxic to the cells and did not improve cardiomyocyte differentiation (e.g. D5, abstract; supplement). Said prior art documents point away from the use of retinoic acid as inducer of cardiogenesis.

Furthermore, the applicant provided additional comparative examples showing a) that hyaluronan esters of retinoic acid are more active than retinoic acid itself, b) that cellular toxicity in the specific range of doses is completely abrogated and c) that mixed hyaluronan

esters of butyric and retinoic acid have an unexpected synergistic effect.

Consequently, an inventive step is acknowledged for claims 1-28 (**Article 33(3) PCT**).

NEW SET OF CLAIMS

1. Use of retinoic esters of hyaluronic acid as stem cells pro-differentiating agents.
2. Use according to claim 1, wherein such esters are characterized in that they have a degree of substitution with retinoic acid is comprised from 0.00001 to 0.5.
3. Use according to claim 2, wherein said degree of substitution with retinoic acid is comprised from 0,002 to 0.1.
4. Use according to claim 1, wherein such esters are mixed esters of hyaluronic acid with butyric and retinoic acids.
5. Use according to claim 4, wherein the mixed esters are characterized in that they have degree of substitution with butyric acid ranging from 0.05 to 1.0, a degree of substitution with retinoic acid ranging from 0.002 to 0.1 and a ratio between the degree of substitution with butyric acid and retinoic acid (DS RA/DS BA) of at least 6.
6. Use according to claim 1, wherein said stem cells are mammalian.
7. Use according to claim 6, wherein said mammalian are chosen among: H. sapiens, primates, higher primates, rodents, swine, bovines.
8. Use according to claims 1-7, wherein said stem cells are of embryonic or somatic origin.
9. Use of esters of hyaluronic acid with retinoic acid for the preparation of medicaments with cardiogenic pro-differentiating activity on stem cells.
10. Use according to claim 9 for preparation of medicaments with a cardiogenic pro-differentiating activity.
11. Use according to claim 10 for preparation of drugs for treatment and prevention of myocardial damages and of cardiomyopathies
12. Use according to claim 11, wherein the myocardial damage is myocardial infarction.
13. Process for in vitro preparation of cardiomyocytes essentially comprising a step of incubation of stem cells with retinoic esters of hyaluronic acid and optionally a selection of the contractile units comprising said cardiomyocytes.
14. Process according to claim 13, wherein said retinoic esters are characterized

by a substitution degree of hyaluronic acid with retinoic acid comprised from 0.00001 to 0.5.

15. Process according to claim 13, wherein such retinoic esters are mixed esters of hyaluronic acid with butyric and retinoic acids.

16. Process according to claim 15, wherein such mixed esters are characterized in that they have a degree of substitution with butyric acid ranging from 0.05 to 1.0, a degree of substitution with retinoic acid ranging from 0.002 to 0.1 and a ratio between the degree of substitution with butyric acid and retinoic acid (DS RA/DS BA) of at least 6.

17. Process according to claim 13, wherein said stem cells are autologous or heterologous.

18. Process according to claim 17, wherein the selection is performed by means of "gene-trapping".

19. Process according to claim 17, wherein said stem cells are chosen among: P19, D3 cells, R1 cells, GTR1 cells.

20. Process for the selection of new molecules with cardiogenic-modulation activity comprising the process according to claims 13-19 and optionally a step for optimization of the selected molecules.

21. Process for preparation of an in vitro cell model for cardiogenic differentiation of stem cells, essentially comprising a step of incubation of said stem cells with retinoic esters of hyaluronic acid alone or in combination with other substances, in suitable culture medium.

22. Process according to claim 21, wherein such retinoic esters are characterized in that they have a degree of substitution of hyaluronic acid with retinoic acid ranging from 0.00001 to 0.5.

23. Process according to claim 22, wherein such retinoic esters are mixed esters of hyaluronic acid with butyric and retinoic acids.

24. Process according to claim 23, wherein such mixed esters are characterized in that they have a degree of substitution with butyric acid ranging from 0.05 to 1.0, a degree of substitution with retinoic acid ranging from 0.002 to 0.1 and a ratio between the degree of substitution with butyric acid and with retinoic acid (DS RA/DS BA) of at least 6.

25. Process according to claim 21, wherein said stem cells are chosen among: P19, D3, R1, GTR1, H1, H7, H9, H9.1 and H9.2 cells.
26. Process according to claim 21, wherein such incubation is followed by a step of selection of the contractile units comprising cells differentiated in cardiomyocytes.
27. A therapeutic method for treating heart failure in a patient in need of such a treatment characterised in that heterologous or autologous stem cells are treated "in vitro" or "ex vivo" with retinoic esters of hyaluronic acid.
28. A therapeutic method according to claim 27 wherein the degree of substitution of hyaluronic acid with retinoic acid is comprised from 0,00001 to 0,5.